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PERMEABILITY OF TROUT ERYTHROCYTES TO NONELECTROLYTES

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A number of years ago M. H. Jacobs made an extensive study of the permeability of erythrocytes of a wide variety of species to various nonelectrolytes (e.g., Jacobs, 1931a, b; Jacobs, Glassman and Parpart, 1935, 1950). Among other things he was interested in the relationship between erythrocyte permeabilities and zoological classification. These studies demonstrated that each class of the vertebrates has characteristic permeabilities which are different from those of other classes. The majority of his data supported the suggestion that the more closely related two species are, the more similar are their permeabilities, but there still are distinctive differences (e.g., Jacobs, Glassman and Parpart, 1938).

The majority of Jacobs' experiments were performed measuring hemolysis times, since the use of photoelectric techniques for following volume changes had not been developed. In addition, the concept of carriers in the red cell membrane had not been well-documented when Jacobs' group was carrying on their experiments, although in their 1950 paper they suggested that some sort of special mechanism might be involved in some cases.

A. K. Solomon's group has made an extensive study of red cell permeability to nonelectrolytes. They are focusing their attention on effective pore radii rather than suggesting that carriers may be involved (*e.g.*, Owen and Solomon, 1972; Galey, Owen and Solomon, 1973; Jennings and Solomon, 1976).

In this laboratory, kinetic studies are being made of the permeability to nonelectrolytes of erythrocytes of vertebrates of the various classes in an attempt to determine the presence or absence of carriers. The present report includes a kinetic analysis, using both a swelling and a shrinking technique, of the permeability of erythrocytes of four species of trout to four nonelectrolytes. There is considerable hybridization with these fishes, but since the results were essentially the same with all of the individuals studied, this should not be a problem.

Materials and Methods

Most of the trout were caught on hook and line but a few were supplied by a hatchery. Blood was obtained by cutting the gills and heparin was the anticoagulant. In most cases the blood was used immediately but in a few instances it was stored overnight in a refrigerator with similar results. Volume changes were measured using a densimeter (cf. Mawe, 1956).

Since the swelling and shrinking experiments were performed during two different summers, there were slight differences in technique. For the swelling experiments, the temperature of the water jacket in the densimeter was maintained by circulating water by gravity at room temperature (about 18° C) which eliminated marked changes in temperature. For the shrinking experiments, water from

a constant temperature water bath (20° C) was circulated through the densimeter water-jacket. A second difference was that whole blood diluted with 0.82% NaCl was used in the former series while cells washed three times in 0.82% NaCl were used in the latter. A solution of 0.82% NaCl (buffered with Tris to pH 7.5) was considered to be isotonic with the bloods (Hoar and Randall, 1969). In all of the experiments the external volume was at least 300 times the volume of cell water, and therefore the external concentration of penetrant can be considered to be constant.

Swelling experiments

For the entrance, or swelling, experiments, the method of Widdas (1954) was used. The rationale behind this method is that as one adds more and more penetrant, if simple diffusion is involved, the rate of penetration will always be proportional to the concentration gradient across the cell membrane, but if facilitated diffusion is involved, the carrier will tend to saturate and so the rate of penetration will eventually fall off. Widdas starts with the basic equation: $dS/dt = K\{(C/C + \phi) - [(S, V)/(S/V + \phi)]\}$, where S is the amount of penetrant in the cell; C, external concentration of penetrant; V, volume of cell water expressed as a fraction of the isotonic volume; ϕ, value for half-saturation of the carrier; and K, a constant. Concentrations and ϕ are expressed in isotonic units (one isotone = the isotonic concentration). If ϕ is large with respect to S and C, Widdas showed that this equation can be simplified and integrated to give: $F(C, V) = kt = C' + 1 - (1 + C)V + (1 + C) \ln [C - C'/(1 - V) (1 + C)]$, where C' is the initial external concentration and k equals K/ϕ. This equation describes the kinetics of simple diffusion. If ϕ is small with respect to S and C, Widdas showed that the following equation can be derived which describes the kinetics of a system with a near-saturated carrier: $F' (C, V) = k't = C(1 + C)\{C' + 1 - (1 + C)V + C \ln [C - C'/(1 - V)(1 + C)\}$, where k' equals $K\phi$.

For a given experiment, values for concentrations and volumes are known and can be substituted in the second and third equations. A table can be constructed showing the numerical values of F and F' (C, V) for several values of V, for each value of C' and C (cf. Widdas, 1954). Such a table has been previously published for a 1 M system (Hunter, 1968) and for an 8.1 M system (Hunter, 1970a) considering that 0.3 M nonelectrolyte is isotonic with mammalian bloods. Since the osmotic pressure of trout blood is slightly less than that of mammalian blood, the solutions used in the present study were 0.91 M thiourea in 0.82% NaCl, 7.38 M urea, ethylene glycol and glycerol in 0.82% NaCl. Since thiourea is not very soluble, it has to be used in lower concentration.

If experimental times plotted against F(C, V) fall on a single straight line and give a family of straight lines when plotted against F'(C, V), this indicates that the kinetics are those of simple diffusion. If the reverse is true, the kinetics are those of a system with a near-saturated carrier.

Details of the experimental procedure are included in the legends to the figures.

Shrinking experiments

For the exit, or shrinking experiments the method of Sen and Widdas (1962) was used. With this method, one measures the rates of exit of a penetrant into

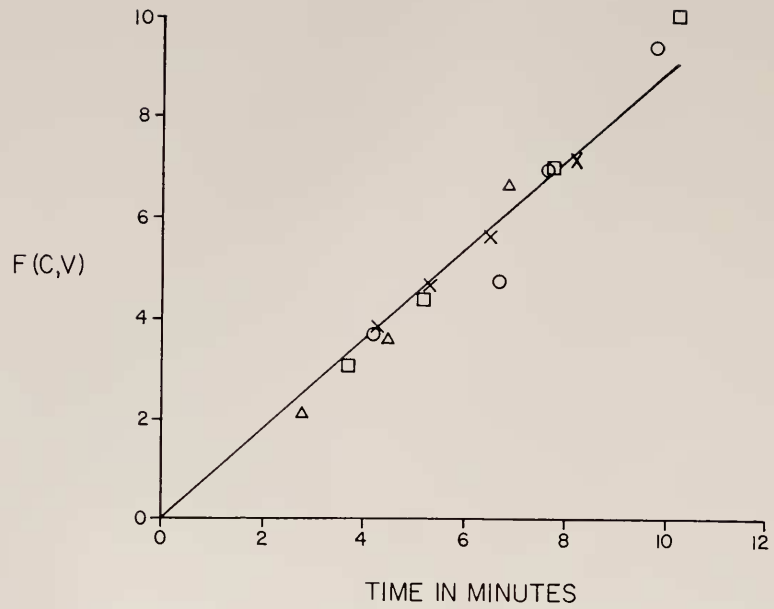

FIGURE 1. Experimental times plotted against F(C, V). Five additions of 0.5 ml each of 7.38 M glycerol in 0.82% NaCl were made to 8.5 ml of German brown erythrocytes in 0.82% NaCl. Additions 2–5 were recorded and are plotted. X's are for an intracellular concentration of 1.50 and an extracellular concentration of 2.84. Circles are for an intracellular concentration of 2.84 and an extracellular concentration of 4.05. Squares are for an intracellular concentration of 4.05 and an extracellular concentration of 5.14. Triangles are for an iiitracellular concentration of 5.14 and an extracellular concentration of 6.14. All concentrations are in isotones.

an external solution which contains increasing concentrations of the same penetrant. If ϕ and concentrations are small, the following relationship can be obtained: $t = (Si + \phi)/K\ (C + \phi)$. This equation tells us that when $t = 0$, $C = -\phi$.

The method for measuring t is given in the legend for Figure 5. Higher external concentrations were not used since the effect is not linear above 63.1 mM (Hunter, Fayad and Mayorga, 1976). Using these times, the value of ϕ is obtained as shown in Figure 6. It should be noted that if only simple diffusion is involved, the straight line as drawn in Figure 6 should be parallel to the x-axis giving a value of ϕ equal to infinity. That is to say, there is no carrier; only simple diffusion is involved.

RESULTS

Typical series of swelling curves and shrinking curves have been published previously (Hunter, 1976). With thiourea, the volume changes resulting from the first two additions of penetrant were too small to measure accurately. With some of the other systems, the first additions of penetrant were made without recording. But in every case, from 3 to 8 swelling curves were available to analyze the kinetics of the system. Figures 1–4 illustrate typical results obtained when experimental times are plotted against either F(C, V) or F′(C, V). The results were the same

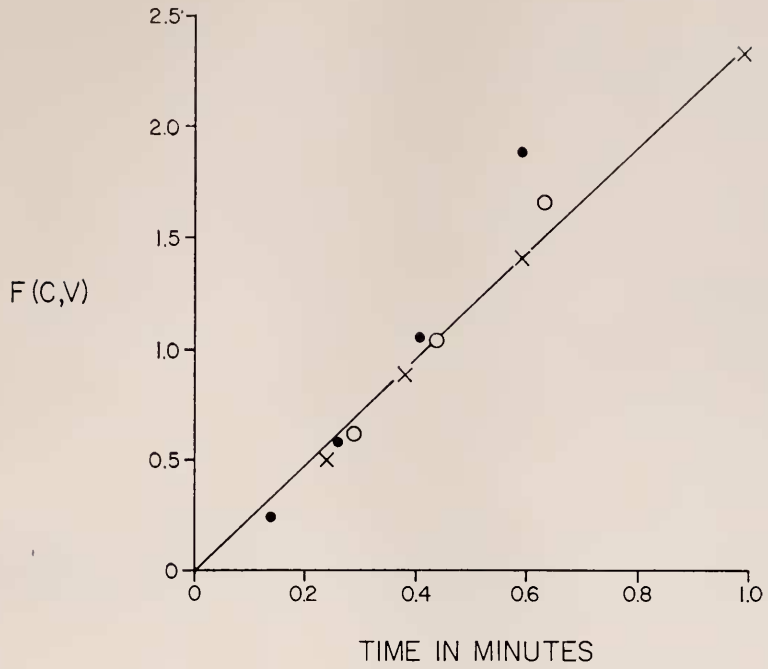

FIGURE 2. Experimental times plotted against F(C, V). Five additions of 0.1, 0.2, 0.4, 0.6 and 0.8 ml of 0.91 M thiourea in 0.82% NaCl were made to 9.9 ml of cutthroat cells in 0.82% NaCl. Only the last three additions were measured. Closed circles are for an intracellular concentration of 0.098 and an extracellular concentration of 0.220. X's are for an intracellular concentration of 0.220 and an extracellular concentration of 0.387. Open circles are for an intracellular concentration of 0.387 and an extracellular concentration of 0.583. All concentrations are in isotones.

with all four bloods and all four penetrants. In all 16 cases the experimental times when plotted against F(C, V) fell on a single straight line and in every case when these same times were plotted against F′ (C,V) a family of straight lines was obtained. In Table I are summarized relative rates of penetration of the four nonelectrolytes into erythrocytes of the four different species of trout.

Graphs were made of the initial portion of each shrinking curve (Fig. 5) in order to measure the time to reach equilibrium volume using the initial, rapid rate of exit. Average values of these times were plotted against external concentrations of penetrants (Fig. 6). These graphs should enable one to distinguish between simple and facilitated diffusion. Least squares regression lines were calculated for each of these graphs to obtain values of the x-intercepts (Table I).

DISCUSSION

Previous work has shown that glycerol, ethylene glycol, thiourea and urea cross the membrane of erythrocytes of several species of mammals and one species of birds by facilitated diffusion (Hunter, 1970a, b; Cainelli, Chui, McClure and Hunter, 1974; Hunter, 1976; Hunter, *et al.*, 1976). The present experiments

TABLE I

Average times in minutes for the erythrocytes to swell to 95% of their original volume and values of the half-saturation constant (ϕ) in millimoles calculated from the data presented in Figure 6 by the method of least squares.

Species	Nonelectrolyte							
	Glycerol		Ethylene glycol		Thiourea		Urea	
	Time	ϕ	Time	ϕ	Time	ϕ	Time	ϕ
Salvelinus fontinalis (brook trout)	5	135	0.2	−379	0.6	∝	4	−646
Salmo gairdneri (rainbow trout)	6	−1,170	0.1	1,364	0.3	∝	2	−1,431
Salmo trutta (German brown trout)	7	42,099	0.2	—	0.6	—	3	—
Salmo clarki (cutthroat trout)	5	−336	0.2	—	0.6	−803	4	534

were undertaken to determine whether or not carriers were involved in the penetration of these four nonelectrolytes into fish erythrocytes. The fact that experimental times obtained from the swelling curves give a single straight line when

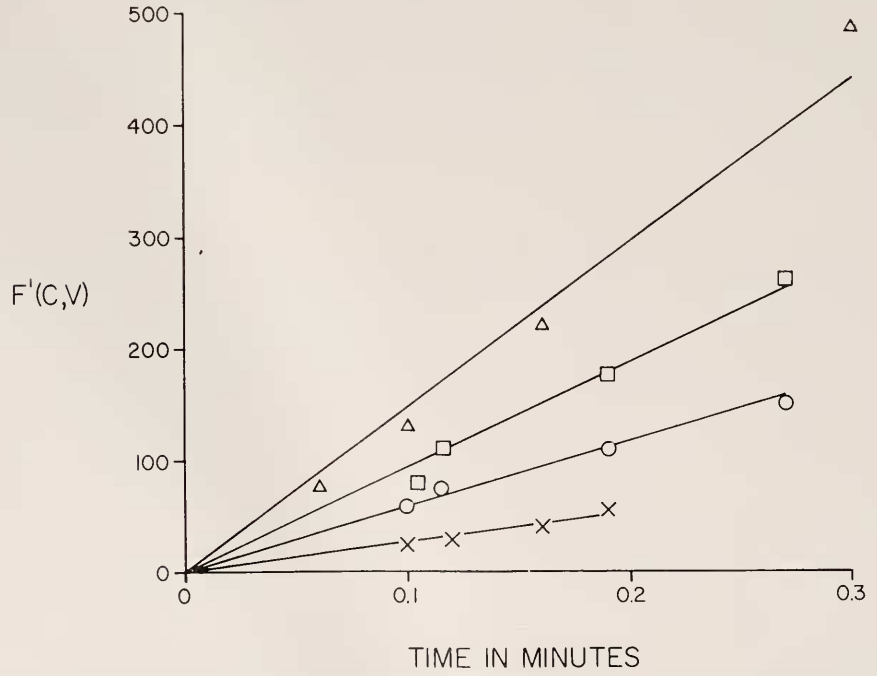

FIGURE 3. Experimental times plotted against F'(C, V) for brook trout blood and ethylene glycol penetration. The procedure was the same as for Figure 1 and the symbols have the same significance.

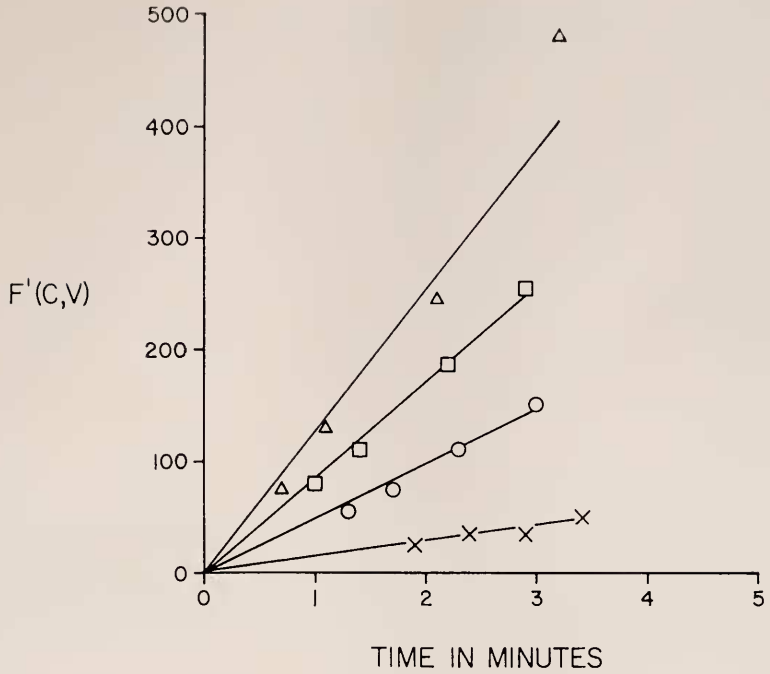

FIGURE 4. Experimental times plotted against F'(C, V) for rainbow trout and urea penetration. The procedure was the same as for Figure 3 and the symbols have the same significance.

plotted against F(C, V) indicates that there is no evidence of saturation. The family of straight lines obtained with the F'(C, V) plot is also evidence of the same thing. From the data presented in Figure 6, it can be seen that in the case of brook trout-thiourea and rainbow-thiourea the three points fall on a straight line parallel to the x-axis, predicting simple diffusion. Except for one or two of the other values in the figure, the three points in each case fall on a line almost parallel to the x-axis, suggesting simple diffusion in each case. From the calculated values for the x-intercept (Table I) it can readily be seen that most of these figures are very large (approaching infinity) and some are even negative. This means that the shrinking data, like the swelling data indicate that these four nonelectrolytes enter the erythrocytes of these four species of fish by simple diffusion (cf., Kaplan, Hays and Hays, 1974).

Comparisons of relative rates of penetration can be made using the numbers in Table I and the values in Figure 6. The absolute values are not directly comparable from the table to the figure since different concentrations of penetrants were used in the two series of experiments. Both sets of data indicate that the rates of entrance and of exit of a given nonelectrolyte are quite similar in these four closely related species. All four species have the highest permeability to ethylene glycol, then thiourea, next urea, with the lowest permeability being to glycerol. This agrees with Jacobs' data (Jacobs, 1931b, 1935).

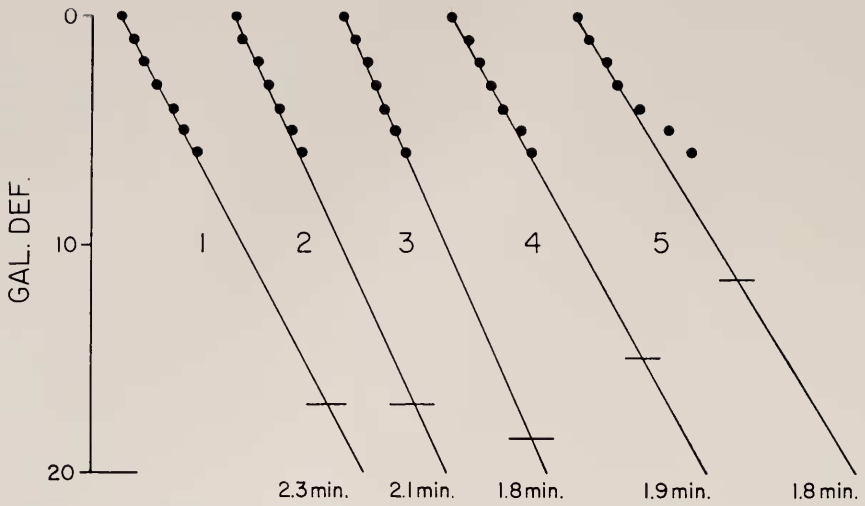

FIGURE 5. Erythrocytes of German brown trout were equilibrated in 200 mM glycerol in 0.82% NaCl (1:10 v/v). 0.25 ml of this cell suspension were added to 10 ml of 0.82% NaCl plus: 1–4.6 mM glycerol; 2–14.3 mM G; 3–23.1 mM G; 4–43.6 mM G; and 5–63.1 mM G. The initial steep portion of each curve is plotted on rectilinear coordinates. Horizontal lines represent equilibrium volume in each case. The times for the lines drawn through the initial points to intersect the horizontal lines were measured using graphs such as these. These times are indicated beside each line.

In mammalian red cells where there is a carrier for urea which also carries thiourea, relative penetration times for these two substances are reversed. Urea penetrates very rapidly and thiourea enters much more slowly. Another interesting comparison is that ethylene glycol enters the cells of the four trout species by simple diffusion much more rapidly than this molecule enters cells of some species of mammals where a carrier is involved. This might suggest that during the course of evolution, with the addition of carriers to the red cell membrane, the movement of urea was speeded up at the expense of a chemical change in the membrane which slowed down the movement of other molecules.

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SUMMARY

Using a densimeter technique, a kinetic analysis was made, employing both entrance and exit studies, of the permeability of erythrocytes of brook trout (*Salvelinus fontinalis*), rainbow trout (*Salmo gairdneri*), German brown trout (*Salmo trutta*) and cutthroat trout (*Salmo clarki*) to glycerol, ethylene glycol, thiourea and urea. All of the data indicate that these four nonelectrolytes cross

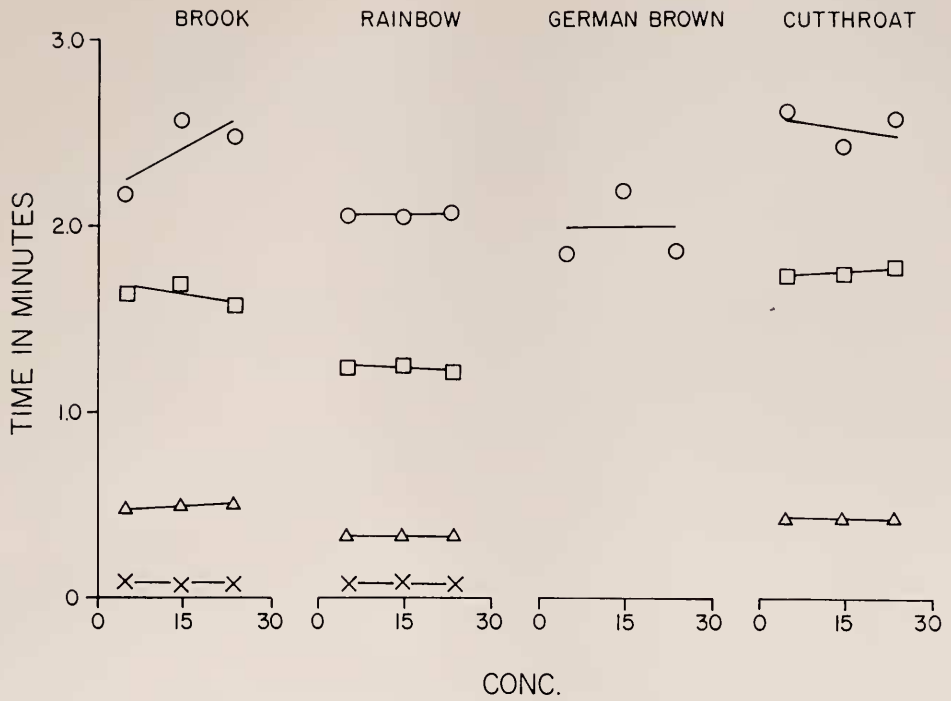

FIGURE 6. Time in minutes for initial tangent to intercept horizontal line drawn through equilibrium volume plotted against calculated external concentration of penetrant. Circles are values obtained with glycerol, X's with ethylene glycol, squares with urea and triangles with thiourea.

the membrane of the erythrocytes of these four species of fishes by simple diffusion only.

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